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Authors: M. A. Abd-El-Hafez, M. D. El-Shafee, S. H. Omar, A. A. Aburahma, S. S. Kamar

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The ameliorative effect of curcumin on cryptorchid and non-cryptorchid testes in induced unilateral cryptorchidism in albino rat: histological evaluation

Running head: Curcumin effect on cryptorchid and non-cryptorchid testes

M.A. Abd-El-Hafez¹, M.D. El-Shafee¹, S. H. Omar¹, A.A. Aburahma², S.S. Kamar¹

¹Department of Medical Histology and Cell Biology, Faculty of Medicine, Cairo University, Cairo, Egypt

²Department of Andrology, Faculty of Medicine, Cairo University, Cairo, Egypt

Address for correspondence: Dr. S.S. Kamar, Department of Medical Histology and Cell Biology, Faculty of Medicine, Cairo University, Cairo, Egypt, tel: +20 01008909069, e-mail: Dr_samaakamar@yahoo.com

The work was conducted in: Faculty of Medicine, Cairo University, Cairo, Egypt.

Abstract

Background: Cryptorchidism, one or both hidden testes, is the most common abnormality of male sexual development. Subfertility or infertility is associated with both unilateral and bilateral cryptorchidism. In this study, we investigate the possible ameliorative effect of curcumin (Cur) on the induced-unilateral cryptorchidism testicular injury in both cryptorchid (Cryp) and non-cryptorchid (non-Cryp) scrotal testes through histological, immunohistochemical and morphometrics.

Materials and methods: 40 adult male albino rats were divided into: control group, Cur control, Cryp group, and Cryp+Cur group. The rat model was surgically established by fixing the left testis in the abdomen. The treated groups were subjected to surgically induced-unilateral cryptorchidism on the left side then were given Cur (80 mg/kg) orally, for 20 days. Histological analysis using hematoxylin & eosin and periodic acid Schiff's reaction was applied. Immunohistochemistry was performed for proliferating cell nuclear antigen (PCNA); to estimate the proliferation in the germinal epithelium, and vimentin; to evaluate Sertoli cells. The results were confirmed by statistical evaluation

of the spermatogenic epithelium height, the seminiferous tubules diameter, the basement membrane thickness, the number of PCNA immunostained cells and the area % of vimentin immunostaining.

Results: distorted seminiferous tubules, substantial degeneration of the germinal epithelium, thickening of the basement membrane with a significant decrease in PCNA and vimentin immunostaining were observed in Cryp group; mainly in the cryptorchid testis. These structural changes were significantly reversed in Crypt+Cur group.

Conclusions: Curcumin proved to be an important and effective medical line for protecting against the unfavorable sequels of cryptorchidism in a rat model.

Key words: testis; unilateral cryptorchidism; curcumin; PCNA; vimentin

INTRODUCTION

Cryptorchidism, one or both hidden testes, is the most common abnormality of male sexual development occurring in about 2.4–5% of full-term newborns [14]. The ratio rises to 30% of premature neonates. Related complications of infertility, malignant transformation, depression and trauma have been recorded [15].

Subfertility or infertility is associated with both unilateral and bilateral cryptorchidism. One-third of unilateral undescended testis complained of fertility impairment with incidence of azoospermia in about 13 % of cases [11]. The impact of cryptorchidism included not only degeneration of germ cells in response to elevated temperature [17] but also increased intratesticular oxidative stress that produced deleterious testicular changes with reduced spermatogenesis [7]. Previous study has demonstrated that unilateral cryptorchidism was associated with increased number of mast cells in both testes, resulting in fibrosis and deterioration of spermatogenesis [1]. The structural defects of both the retained and scrotal testis have been concluded in other studies [35]; [15].

The concept of ‘early orchidopexy’ has established as the primary treatment for cryptorchidism. However, orchidopexy alone is insufficient to completely restore spermatogenesis and there is a domain for a germinal epithelial protective substance [6]. Human spermatogonia from Cryp patients can piecemeal differentiate into haploid

spermatids when treated with retinoic acid and stem cell factor [36]. Antioxidants have proved to significantly increase the sperm count and germ cell count in Cryp rats [3].

Curcumin (Cur), the active ingredient of the dietary spice turmeric (*curcuma longa*), is widely used in medical practice. Its efficacy is due to its phenolic group [33]. It is known to be anti-inflammatory, antineoplastic, cardioprotective and renoprotective reagent [36]. Moreover, it has bi-functional antioxidant effects by protecting the cell against the reactive species and stimulating up regulation of cytoprotective proteins [34].

The aim of our study is to evaluate the possible ameliorative effect of Cur on the induced unilateral cryptorchidism in both Cryp and non-Cryp testes.

MATERIALS AND METHODS

Experimental design

This study included 40 adult male albino rats, each of 200–250 gm body weight. The animals were bred in the Animal House of Faculty of Medicine, Cairo University. Each group, or subgroup, was kept in separate wire cage at room temperature, fed ad libitum with free water supply. All procedures were held under ethical guidelines of Animal Care and Use Committee of Cairo University. The rats were divided equally into 4 groups (n=10):

Control group (GpI): The rats were equally subdivided into 2 subgroups (n=5):

- *Blank control (GpIa)*: the rats were not exposed to any surgical procedure.
- *Sham (GpIb)*: subjected to sham operation on day 1 of the experiment. Under anaesthesia and complete aseptic conditions, a lower midline abdominal incision was performed. The left testis was displaced into the abdomen then replaced again into the scrotal sac, then the incision was closed. The rats were administered buprenorphine 0.05 mg/kg by intraperitoneal injection/8 hours; as post-operative analgesic for 7 days. Besides, they were given 1 ml dimethyl sulfoxide (DMSO) orally once daily for 20 days.

Cur control group (GpII): were given Cur at a dose of 80 mg/kg, dissolved in 1ml DMSO, once daily orally, for 20 days.

Cryp group (GpIII): rats were subjected to surgically induced-unilateral cryptorchidism on the left side. Animals were subjected to the same surgical procedures as **GpIb** but the left testis was displaced into the abdomen and fixed. The gubernaculum on the left side was

separated, the testis was displaced into the abdomen, and the inguinal canal was closed [7]. The incision was sutured and the rats were administered post-operative analgesic. The animals were left for 20 days after surgery without any treatment [22].

Cryp+Cur group (GpIV): rats were subjected to surgically induced-unilateral cryptorchidism on the left side as described in **GpIII** then were given Cur orally, for 20 days.

All rats were euthenized at the end of the experiment by intraperitoneal injection of thiopental sodium (50mg/kg). Testis specimens were fixed in bouin solution and embedded in paraffin. Serial sections of 5µm thickness were cut and subjected to histological and immunohistochemical studies.

Histological studies

Hematoxylin and eosin stain (HE) to illustrate the morphological change and Periodic acid Schiff's (PAS) reaction to demonstrate the basement membrane.

Immunohistochemistry for

Proliferating cell nuclear antigen using a mouse monoclonal antibody (Ab) [Thermo Scientific Laboratories, USA, Cat.# MS106P] as a criterion for the proliferating cells.

Vimentin intermediate filament using monoclonal antibody (Cell Marque Corporation, Toll-Free North America, Cat.# 347M-18).

Morphometrics

Employing "Leica-Qwin 500 C" image analyzer (Cambridge, England), ten non-overlapping fields/rat testes were examined and the following parameters were estimated:

- Height of the spermatogenic epithelium (SEp) and the diameter of the seminiferous tubules in HE sections.
- Thickness of the basement membrane in PAS stained sections.
- Number of PCNA positive (+ve) immunostained cells.
- Area % of vimentin +ve immunostaining.

Statistical studies

The estimated measurements were compared and analyzed using one way analysis of variance of SPSS software version-19. Comparison between the different groups was followed by Post-Hoc, Tukey test. Quantitative representative data was obtained and summarized as means \pm standard deviations (SD). Probability (P) values <0.05 were considered statistically significant.

RESULTS

Clinical observation: no mortality was recorded in the experimental rats and no changes were noted in their behaviours in water and food consumption.

HE stain: testicular sections of control rats and rats from GpII displayed normal architecture of the seminiferous tubules and the interstitial tissue (IS) (Figure 1a,1b). The Cryp testes of GpIII revealed severely distorted seminiferous tubules and partial separation of the basement membrane in some areas. In most of the examined fields, there was obvious degeneration of the germinal epithelium with some shed cells in the lumen. The non- Cryp testes from GpIII showed mild disorganization of the seminiferous tubules. The lining epithelium showed spermatogonia, primary spermatocytes with absence of late stage of germ cells (Figure 1c,1d). The GpIV revealed obvious protection of the abdominal and scrotal testes. There were normal structure of the seminiferous tubules containing spermatozoa in the lumen and IS in both abdominal and scrotal testes. Seminiferous tubule displayed all germinal cell layers with sperm in the lumen (Figure 1e,1f).

PAS stain: sections from GpIII revealed strong thick PAS +ve-reaction in the thick irregular basement membrane in both scrotal and abdominal testes. In GpIV, thin strong PAS +ve-reaction in the basement membrane of both abdominal and scrotal testes was illustrated (Figure 2).

Immunohistochemistry (Figure 3):

Using PCNA immunostaining, the Cryp testes from GpIII showed few +ve PCNA immunostained cells near the basement membrane in severely degenerated SEp. The non-Cryp testes revealed many +ve PCNA immunostained cells in the early stages of the SEp and weak or absent immunostaining in the late stages of the spermatogenic cells. Group IV revealed diffuse +ve PCNA immunostaining in both testes.

In vimentin immunohistochemistry, Cryp testes of GpIII showed +ve vimentin immunostaining in Sertoli cells in the perinuclear region. The right scrotal testes of the same rats revealed +ve vimentin immunostaining mainly in the perinuclear region of Sertoli cells with few +ve apical immunostaining. In GpIV, both testes revealed numerous +ve vimentin immunostaining of Sertoli cells in the perinuclear regions and throughout the cytoplasm extending into the apices.

Statistical Analysis (Table-1):

The height of the SEp and seminefrous tubules diameter: GpIII showed a significant decrease in both parameters as compared to GpI, GpII, and GpIV. Meanwhile, both parameters in the Cryp testes of GpIII were significantly decreased as compared to the scrotal testis of GpIII. In GpIV, the mean height of SEp and the mean diameter of seminiferous tubules in the Cryp testes were significantly decreased as compared to GpI, GpII and non-Cryp testes of GpIV.

The thickness of the basement membrane: in both testes of GpIII, the mean thickness of the basement membrane displayed a significant increase as compared with GpI, GpII, and GpIV. In addition, the mean thickness of basement membrane in Cryp testes of GpIII was significantly increased compared to the non-Cryp testes of GpIII. The mean thickness of basement membrane in Cryp and non-Cryp testes of GpIV were comparable.

The mean cell count of PCNA +ve immunostained cells: in both testes of GpIII, the mean cell count of PCNA +ve immunostained cell was significantly decreased as compared to GpI, GpII and GpIV. Besides, it was significantly decreased in the Cryp testes of GpIII as compared to the non-Cryp testes of GpIII. The mean cell count of PCNA +ve immunostained cell in the Cryp testes of GpIV was significantly decreased as compared with GpI, GpII and non-Cryp testis of GpIV.

The mean area % of vimentin immunostaining: both testis of GpIII showed a significant decrease as compared to GpII and GpIV. In the abdominal testes of GpIV, it was significantly decreased as compared to GpI, GpII and scrotal testes of GpIV.

DISCUSSION

Cryptorchidism is a common congenital malformation in the male reproductive system. It is documented to have long-term sequels such as infertility, depression, and testicular cancer. The impacts included degeneration of germ cells in response to elevated temperature [17] and increased intratesticular oxidative stress [7]. The objectives for cryptorchidism management are to preserve fertility and ameliorate the risk of malignancy [29].

Experimentally induced unilateral cryptorchidism is stellar method to study undescended testis in relevance to spermatogenesis against temperature gradient in both testes [12]. In the present work, cryptorchid testes of GpIII revealed severely distorted seminiferous tubules and SEp. The non-Cryp testes of GpIII displayed mild disorganization of the seminiferous tubules with absence of sperm in most of fields. However, a significant difference in the height of germinal epithelium and the diameter of seminiferous tubules was noted between the Cryp and the non-Cryp testes of GpIII. These findings were in accordance with previous study of Moon et al., [25]. It was postulated that histological changes associated with cryptorchidism resulted in a significant reduction in the number and the diameter of seminiferous tubules with amelioration of the number and proliferation of spermatogonia. Besides, most of the proliferating cells detected were Sertoli cells suggesting increased risk of Sertoli cell tumors. Also, structural defects of both the retained and scrotal testes were reported [35].

High temperature was suggested to induce disruption of spermatogenesis in cryptorchid testis [12]. Lin et al., [20] found that hyperthermia initiates oxidative stress and apoptosis in spermatogenic cells with subsequent affection of fertility. This was explained by Tekayev et al., [32] that testicular tissues are rich in polyunsaturated fatty acids and poor in antioxidant defense. Thus, they are prone to be attacked by reactive oxygen species (ROS) which are able to oxidize proteins, lipids and deoxyribonucleic acid leading to cellular damage. The equalization between production and clearance of ROS provides an important role in the spermatogenesis as physiological level of ROS maintains the body's normal physiological functions, whereas excessive ROS can cause apoptosis.

Interestingly, Acikgoz et al., [1] found that unilateral cryptorchidism was associated with increased number of mast cells in both testes, resulting in deterioration of spermatogenesis. According Aydin et al., [5], the unilateral cryptorchidism causes endocrine dysfunction in the body, influencing the secretion of sex hormone and occurrence of allergic reaction. Recent study has revealed that cryptorchidism leads to hypothalamic-pituitary-gonadal dysfunction, which was assumed to interfere with the contralateral testicular function and morphology [30]. Moreover, it was documented that affection of sensory branch of genito-femoral nerve is a finding in cryptorchidism [17]. The abnormal environment in the Cryp testis has deleterious effects on the genito-femoral nerve that induces changes in blood circulation and the microenvironment in the contralateral non-Cryp testis [26].

Rats from GpIV that received curcumin treatment after induction of unilateral cryptorchidism revealed obvious protection of the abdominal and scrotal testes. They exhibited normal architecture of the seminiferous tubules and SEp. This result was approved by the significant increased in the mean height of germinal epithelium and the mean diameter of the seminiferous tubules as compared to GpIII. However, the means in the non-Cryp testes of GpIV was comparable to GpI and significantly increased as compared to the Cryp testes of GpIV. Thus, indicating substantial protective effect of Cur in the Cryp testis and full protection in the contralateral non-Cryp testis.

In the current study, GpIII displayed significantly thickened irregular basement membrane in both testes as compared to GpI. However, GpIV revealed thin basement membrane of both testes that was comparable to GpI. In the study of Hassanin et al., [16], the thickening of the basement membrane was detected and explained by the harmful effect of oxidative stress on the testis induced by acrylamide. The protective effect of Cur could be referred to its antioxidant effect similar to the antioxidant vitamin E that protected the testis from the oxidative stress.

In PCNA immunohistochemistry, control rats illustrated diffuse nuclear immunostaining in the spermatogenic cells in addition to the IS. Thus, indicating healthy high proliferative capacity of the testicular tissue [28]. In GpIII, significant decrease in PCNA immunostained cells was noted as compared to GpI. If any in the cryptorcid testes, the PCNA immunostained cells were noted near the basement membrane. The scrotal testes showed +ve PCNA immunostained cells, mainly in the

early stages of the spermatogenic cells and weak or absent PCNA immunostaining in the late stages. It was reported that the testicular tissues obtained from rats treated with cadmium had a harmful effect on the testis through its oxidative stress activity [27]. This was supported by Dutta et al., [12] who proved that oxidative stress ameliorate the proliferation and induces apoptosis in the highly differentiated spermatogenic cells causing its degeneration in the order of; sperms, spermatids, spermatocytes then spermatogonia.

Testicular sections of rats from GpIV revealed substantial +ve PCNA immunostaining in both abdominal and scrotal testes. However, this significant preservation of the proliferative capacity was still partial in the Cryp testes of GpIV as compared to the scrotal testes of the same group. This is in agreement with Yang et al., [37] who reported that human spermatogonial stem cells from cryptorchid patients can progressively differentiate into haploid spermatids when treated with the antioxidant retinoic acid and stem cell factor. Previous studies proved that antioxidants significantly increase the sperm count and germ cell count in cryptorchid rats [2]; [3].

In Addition to antioxidant role, it was indicated that curcumin enhances the proliferation, stemness and colony formation in dose dependent manner. In small doses, it increased the expression of marker proteins coupled with the cell growth, telomerase activity and stemness acting signaling pathways [19]. Furthermore, it was reported that curcumin is a favorable anticancer drug due to its beneficial induction of proliferation arrest and cell death in a variety of tumor cells through down-regulation of specific proteins [18]. According to the study of Cao et al., [8] Cur inhibited cancer cell proliferation and augmented apoptosis of osteoclastoma cells via repression of matrix metalloproteinase-9 and nuclear factor kappa beta, and stimulation of c-Jun N-terminal kinases signaling pathways. In addition to the study of Srivastava et al., [31], it synergistically modulated Wnt/ β -catenin signaling pathways and possessed anti-proliferative activity in multiple cancer cell lines. All these findings support the advantageous use of Cur in cryptorchidism, not only to enhance the stemness and proliferation of spermatogonia but also to protect against cancer development.

Vimentin immunohistochemistry in control rats illustrated +ve immunostaining of Sertoli cells basally with characteristic apical projections. Vimentin is an intermediate filament detected in mature Sertoli cells. Its distribution pattern is

harmonic with its pivotal role in maintaining tissue integrity and preservation of spermatogenesis. It radiates apically in the cytoplasm to become attached with the specialized membrane junctions, desmosome-like junctions that connect germ cells with Sertoli cells [24].

Cryptorchid testes of GpIII showed substantial reduction in the vimentin immunostaining; mostly detected basally around nuclei of Sertoli cells indicating collapse of the vimentin intermediate filaments and their disorganization in the basal region of the Sertoli cells. The scrotal testes of the same rats revealed few apical vimentin immunostaining besides the perinuclear region denoting partial separation of vimentin away from the plasma membrane [13]. In agreement, Mohammed et al., [23] detected significant decrease of vimentin immunostaining in astaxanthin induced-testicular damage. The damage was triggered by chronic stress through excessive production of free radicals.

It was reported that cytoskeleton, adherence proteins and cellular adhesion molecules functionally work inter-dependently rather than independently in the homeostasis of spermatogenic cellular junctions [11]. Besides, the disrupted inter-Sertoli germ cell junctions have been demonstrated to cross talk with the defective spermatogenesis in cryptorchidism [9]. On the other hand, the anchoring junction (An-J) proteins are involved in the regulation of germ cell apoptosis. They can disrupt vimentin filaments at the site of Sertoli germ cell An-J, thus inducing up-regulation surge of the testicular Fas-receptor with subsequent germ cell apoptosis [38].

In GpIV, the mean area % of vimentin immunostaining was significantly increased as compared to GpIII. However, the Cryp testes of GpIV displayed significant difference as compared to GpI and scrotal testes of GpIV. These findings signalize that curcumin is capable of preventing disaggregation of Sertoli germ cells contacts and spermatogenic cell apoptosis that was induced by hyperthermia [21].

Limitation of the study

The limitations of this work were the genetic and the hormonal factors that should be included in the estimation of *curcumin* on the unilateral cryptorchidism for upcoming clinical trails.

CONCLUSIONS

In conclusion, curcumin proved to be an important and effective medical line for protecting against the unfavorable sequels of cryptorchidism parallel to orchiectomy. It is one of the antioxidants that improve the fertility after surgery with potential role to protect against cancer transformation.

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Table 1. Curcumin significantly protected the testicular tissue and improved the germinal epithelium proliferation in both cryptorchid and non-cryptorchid testes

Groups	Mean height of SEp (μm)	Mean diameter of seminiferous tubule (μm)	Mean diameter of the basement membrane thickness (μm)	Mean cell count of PCNA immunostained cells	Mean area % of vimentin immunostaining
GpI	97.01 \pm 5.64	362.27 \pm 16.48	0.85 \pm 0.15	36.7 \pm 2.93	26.2 \pm 3
GpII	97 \pm 5.47	357.29 \pm 10.13	0.9 \pm 0.13	38.1 \pm 2.92	26.23 \pm 2.66
Cryp GpIII	9.18 \pm 2.25*!#	141.94 \pm 14.62*!#	5.61 \pm 0.61*!#	6.7 \pm 1.49*!#	10.89 \pm 2.09*#
Non-Cryp GpIII	69.87 \pm 7.49*#	202.93 \pm 23.96*#	4.67 \pm 0.72*#	11.6 \pm 2.01*#	11.51 \pm 1.76*#
Cryp GpIV	89.29 \pm 2.38*\$	297.12 \pm 14.99*\$	1.13 \pm 0.32	32.1 \pm 2.08*\$	20.28 \pm 2.37*\$
Non-Cryp GpIV	95.90 \pm 3.89	348.62 \pm 14.58	0.84 \pm 0.15	36.6 \pm 2.07	24.3 \pm 2.05

Data presented with means \pm SD. * Significant as compared to GpI; ! significant as compared to Cryp GpIII; # significant as compared to Cryp and non-Cryp GpIV; \$ significant as compared to non-Cryp GpIV.

Figure 1. HE stained rat testes (x200) showing: (A) GpI and (B) GpII: the seminiferous tubules containing spermatozoa (star) in the lumen, the IS e harbouring clusters of Leydig's cells (L), the SEp resting on basal lamina with Sertoli cells (arrowheads), attached mature spermatids (curved arrows), numerous spermatogonia (arrows) and primary spermatocytes (Ps). **(C) Cryptorchid testis in GpIII:** substantial distortion and collapse of the seminiferous tubules with partial separation of the basement membrane (wavy-arrow), obvious degeneration of the germinal epithelium. Some cells are sloughed in the lumen (bifid-arrow). **(D) Non-Cryp testis in GpIII:** mild

disorganization of the seminiferous tubules with expansion of interstitial space, the SEp illustrating Sertoli cells (arrowhead), spermatogonia (arrows), primary spermatocytes (Ps) and absence of sperms. **(E)** Cryptorchid and **(F)** Non-Cryp testes in **GpIV**: normal structure of the seminiferous tubules with all germinal cell layers, mature spermatids and spermatozoa (star) in the lumen, and IS containing clusters of Leydig's cells (L).

Figure 2. PAS stained rat testes (x400) showing: (A) GpI and (B) GpII: thin strong PAS +ve-reaction in the basement membrane (arrows). **(C)** Cryptorchid and **(D)** Non-Cryp testes in **GpIII**: strong PAS +ve-reaction in the thick irregular basement membrane (arrows). **(E)** Cryptorchid and **(F)** Non-Cryp testes in **GpIV**: thin strong PAS +ve-reaction in the basement membrane (arrows).

Figure 3. PCNA and vimentin immunohistochemically stained rat testes (x400).
PCNA immunohistochemistry showing: (A) GpI: and **(C) GpII:** diffuse +ve PCNA immunostaining in the nuclei of spermatogenic cells (arrow) and in the IS (star). **(E)** Cryptorchid testis in **GpIII**: few +ve PCNA immunostained cells (arrow) near the basement membrane in severely degenerated SEp. **(G)** Non-Cryp testis in **GpIII**: many PCNA immunostained cells in the early stages of the spermatogenic cells (arrow) with weak (curved-arrow) or absent (arrowhead) immunostaining in the late stages of the spermatogenic cells. **(I)** Cryptorchid and **(K)** Non-Cryp testes in **GpIV**: diffuse +ve PCNA immunostaining in the nuclei of spermatogenic cells (arrow). **Vimentin immunohistochemistry showing: (B) GpI and (D) GpII:** +ve vimentin immunostaining of Sertoli cells in the perinuclear region (arrows), throughout the cytoplasm (bifid-arrow) and apically from nucleus (wavy-arrows). Positive immunostaining is noted in myoid cells (arrowhead), endothelium (curved-arrow) and IS cells (star). **(F)** Cryptorchid testis in **GpIII**: +ve vimentin immunostaining around the nuclei of Sertoli cells (arrows), in myoid cells (arrowhead) and IS cells (star). **(H)** Non-Cryp testis in **GpIII**: +ve vimentin immunostaining mainly in the perinuclear region of Sertoli cells (arrows) with few +ve immunostaining extending through the cytoplasm apically (wavy-arrow). **(J)** Cryptorchid and **(L)** Non-Cryp testes in **GpIV**: +ve vimentin immunostaining of Sertoli cells in the perinuclear region (arrows), throughout the cytoplasm (bifid-arrow) and apically from nucleus (wavy-arrows).





